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# Expression of an Immunogenic Protein derived from Spike Protein of Porcine Epidemic Diarrhea Virus in two *E. coli* Strains

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## ABSTRACT

*Escherichia coli* is recognized as a fast, easy and inexpensive host system for production of valuable proteins including subunit proteins for vaccination. In this study, we studied whether PEDVSME, a new immunogen designed from spike protein of PEDV, can be produced in *E. coli* by studying in two strains: BL21(DE3) and BL21(DE3)pLysS. The prokaryotic expression plasmid, pET28b, was generated to contain the *PEDVSME* gene. Recombinant clone of pET28b.PEDVSME was successfully generated and selected in *E. coli* DH5α. Two strains of *E. coli*, BL21(DE3) and BL21(DE3)pLysS, were then transformed with recombinant plasmid pET28b.PEDVSME. Expression of the *PEDVSME* gene was induced with 1 mM IPTG. Analyzed by SDS-PAGE and Western blot, PEDVSME was not detected in *E. coli* BL21(DE3) but highly expressed in strain BL21(DE3)pLysS. Importantly, although the PEDVSME protein could be observed in soluble form, majority of the protein was found in insoluble form (inclusion body). This work provides useful information regarding appropriate *E. coli* host strain for efficient production of the immunogen PEDVSME, bringing a feasibility of using PEDVSME as a subunit vaccine.

**Keywords**: *E. coli*, PEDV, Protein expression, Spike protein, Vaccine

## INTRODUCTION

Porcine Epidemic Diarrhea Virus (PEDV) is worldwide known as the etiological agent that causes acute disorder, so-called Porcine Epidemic Diarrhea (PED), with the main symptoms expressing as severe vomiting and diarrhea in swine (Lee, 2015). PEDV, which is classified as a member of the *Coronaviridae* family, is an enveloped virus consisting of approximately 28-kb long, positive sense ssRNA genome with a 5′ cap and a 3′ poly (A) tail (Lee, 2015). The outbreaks of PEDV infection cause a very high mortality rate (up to 100%) in pigs, especially piglets, resulting in a large economic burden on pork producers around the world (Song, 2015; Tian et al., 2016).

The glycosylated spike (S) protein of PEDV has been reported as a crucial antigen for vaccine development because it is the main mediator in facilitating the binding between virus and the host cell surface receptor during the virus infection (Lin et al., 2016; Okda et al., 2017). However, previous studies have demonstrated that a recombinant DNA vaccine expressing full-length S protein or truncated S protein (S1 protein) could not provide cross protection among different PEDV strains (Meng et al., 2013; Wang et al., 2015) and also did not transfer complete passive immunity in piglets from vaccinated sows (Makadiya et al., 2016). Moreover, S protein is originally huge in size, approximately 180 kDa, and undergoes post-translational modification (Song & Park, 2012), leading to a substantial issue regarding potent protein expression.

Core of neutralizing epitope domain (CO-26K equivalent, COE) of PEDV has been identified within the spike (S) protein with ability to induce neutralizing antibody (Chang et al., 2002). Since then, COE has become the main target for PEDV vaccine development. COE-based vaccines have been shown to be immunogenic and capable of inducing antibodies against PEDV (Cruz et al., 2006; Okda et al., 2017). However, antibodies induced by COE-based vaccines could not provide complete PEDV neutralization (Chang et al., 2002). In addition to COE domain, other neutralizing epitope regions were later discovered. In 2006, 2C10 (GPRLQPY) region at the C-terminal of the S

protein was identified (Cruz et al., 2006; Cruz et al., 2008) and later in 2008, the SS2 (748YSNIGVCK755) and SS6 (764LQDGQVKI771) located at the S2 region of the S protein were identified (Sun et al., 2008). The latest neutralizing epitope called peptide M (MQYVYTPTYYML) located in N-terminus of S protein was identified in 2015 (Cao et al., 2015). Therefore, designing of PEDV vaccine by conjugating COE together with the four known neutralizing epitopes as antigen should give a more potent vaccine that is capable of inducing neutralizing antibodies against epitopes on both S1 and S2 domains. With the aim to enhance immunogenicity and efficacy of PEDV vaccine, we have recently designed and developed a new vaccine immunogen, named PEDVSME.

A vaccine can be developed using multiple different platforms. Previously, PEDVSME-based vaccines have been developed based on 2 platforms: plasmid DNA and adenovirus. To further develop PEDVSME as a subunit vaccine, the PEDVSME protein must be produced in large amount. Moreover, the PEDVSME protein is also needed for measurement of the immune responses in vaccinated animals using immunological assays such as ELISA, ELISPOT and intracellular cytokine staining (ICS). To achieve large-amount protein production, we then employed prokaryotic expression system for PEVSME protein production.

*Escherichia coli* is by far well-known as a fast, easy and inexpensive expression platform for biopharmaceutical productions including subunit proteins for vaccination. *E. coli* has become the most popular expression platform due to its rapid growth (approximately 20 minutes for the cell doubling time), high safety approved by Food and Drug Administration (FDA) for human application, low expense involving with types of media usage, and common growth condition (37 °C) (Rosano & Ceccarelli, 2014; Sørensen & Mortensen, 2005). However, there are numerous strains of *E. coli* employed as an expression host. Among these, two strains, BL21(DE3) and BL21(DE3)pLysS are widely used. Importantly, a proper selection of expression strains is profoundly important for efficient production of heterologous recombinant proteins.

In this study, we aimed to produce the PEDVSME immunogen in *E. coli*, a prokaryotic host system. Two *E. coli* strains, BL21(DE3) and BL21(DE3)pLysS, were used as cell factories to express the PEDVSME

immunogen. Protein expression was then investigated using SDS-PAGE and Western blot.

## MATERIALS AND METHODS

### Bacterial strains, plasmid and *PEDVSME* gene

*E. coli* DH5α was used as the cloning host for recombinant clone selection and amplification of expression vector. Two different *E. coli* expression strains, BL21(DE3) and BL21(DE3)pLysS, were used as hosts for protein expression. All host cells were grown in Luria Bertani-miller medium (Himedia, India).

The plasmid, pET28b (Invitrogen) were utilized as an expression vector in prokaryotic host cells. The gene encoding PEDVSME was synthetically prepared (Integrated DNA Technologies, Inc. (Singapore)).

### Construction of recombinant plasmid harboring *PEDVSME*

The DNA fragment of *PEDVSME*, a gene that is codon-optimized with pig's codon usage, was released from pUCIDT plasmid by digesting with *Hind*III and *Not*I ligase (NEB, England). The gene fragment was then ligated into the corresponding site of the expression plasmid pET28b using T4 DNA ligase (Thermo Fisher Scientific Inc., USA). Chemically competent *E .coli* DH5α was transformed with the ligation mixture using heat shock method and plated on LB agar supplemented with Kanamycin (50 µg/ml) (AppliChem, Germany). Subsequently, recombinant clones were screened using rapid size screening. Selected recombinant clones were culture in LB broth and subjected to plasmid extraction. Restriction enzyme digestion with *Hind*III and *Not*I was performed to confirm the presence of the gene fragment in the selected recombinant clones. Nucleotide sequence in each clone was then confirmed by automate DNA sequencing.

### Generation of *E. coli* BL21(DE3) and BL21(DE3)pLysS carrying *PEDVSME* and induction of gene expression

Two strains of *E. coli,* BL21( DE3) and BL21( DE3) pLysS, were transformed with recombinant plasmid pET28b.PEDVSME using heat shock technique, followed by spreading on LB agar containing kanamycin ( 50

μg/ml). In parallel, BL21(DE3)pLysS was also transformed with the empty plasmid pET28b and resulting transformant was used as a negative control. Two colonies from each strain were inoculated into Luria-Bertani (LB) medium with 50 μg/ml kanamycin and grown at 37 °C with shaking speed of 200 rpm for overnight. The culture was diluted (1:40) in 4 ml of fresh LB medium and incubated at 37 °C (200 rpm). When the optical density at 600 nm ($OD_{600}$) reached 0.6 per milliliter (ml), Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. The cultures were further incubated for 3 hours at 37 °C with shaking at 200 rpm. Protein was extracted using CelLytic™ B Plus kit (Sigma-Aldrich, Inc., USA) according to the manufacturer's instructions. Protein samples were collected as soluble (supernatant fraction) and insoluble fractions (pellet fraction). Protein expression was then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot.

**SDS-PAGE and Western blot**

Protein samples were mixed with 4x sample loading buffer (150 mM Tris-HCl, pH 6.8, 35% glycerol, 10% SDS, 4% Triton X-100, 400mM DTT, 0.4% bromophenol blue) to a final concentration of 1x and then heated at 98 °C for 5 minutes. Proteins were then separated on 10% SDS-PAGE and stained with InstantBlue™ (Expedeon, UK) or transferred by electroblotting onto a nitrocellulose membrane (Bio-Rad, Germany) using Trans-Blot® SD Semi Dry Electrophoretic Transfer Cell (Bio-Rad, USA) according to the manufacturer's instructions. The membrane was blocked with PBS containing 5% (w/v) skim milk and 0.2% (v/v) Tween 20 (5% PBST) overnight at 4 °C and subsequently incubated with a 1:2000 dilution of mouse monoclonal anti-V5 antibody (Ebioscience, USA) in 5% PBST for 2-3 hours at room temperature. Membranes were washed three times with PBS containing 0.2% (v/v) Tween 20 (PBST), followed by incubation with a 1:10,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Abcam, USA) in 5% PBST for 1-2 hours at room temperature. Membranes were washed three times with PBST and developed in 1-Step™ Ultra TMB-Blotting solution (Thermo Fisher Scientific Inc., USA) for color development.

## RESULTS

### Generation of recombinant plasmid harboring *PEDVSME*

PEDVSME is our new immunogen, which was designed to contain COE and multiple neutralizing epitopes. The nucleotide sequence of the *PEDVSME* gene is codon-optimized with pig's most frequently used codons and was synthetically made. To produce the PEDVSME immunogen in *E. coli*, a prokaryotic expression plasmid pET28b was employed.

The *PEDVSME* gene fragment was obtained from the pUCIDT plasmid by digestion with *Hind*III and *Not*I. Enzymatic digestion result showed the linearized vector and the *PEDVSME* fragment at the expected sizes of 2752 bp and 948 bp, respectively (Figure 1A). The gene fragment was ligated into plasmid pET28b at *Hind*III and *Not*I sites. Following the transformation of *E. coli* DH5α with ligation mixture, clones carrying recombinant plasmid were screened using rapid size screening. Based on the size of the plasmid in comparison to the size of the original plasmid pET28b, several clones were seen to contain foreign gene (Figure 1B). Two recombinant clones were then opted for plasmid extraction. The presence of the *PEDVSME* gene fragment in selected recombinant clones was verified by digestion with *Hind*III and *Not*I. The digested product was seen at the expected size of 948 bp (Figure 1C), confirming the presence of the *PEDVSME* gene fragment in the pET28b plasmid. Moreover, DNA sequencing confirmed that the nucleotide sequence of the *PEDVSME* gene in recombinant clones contain no mutation.

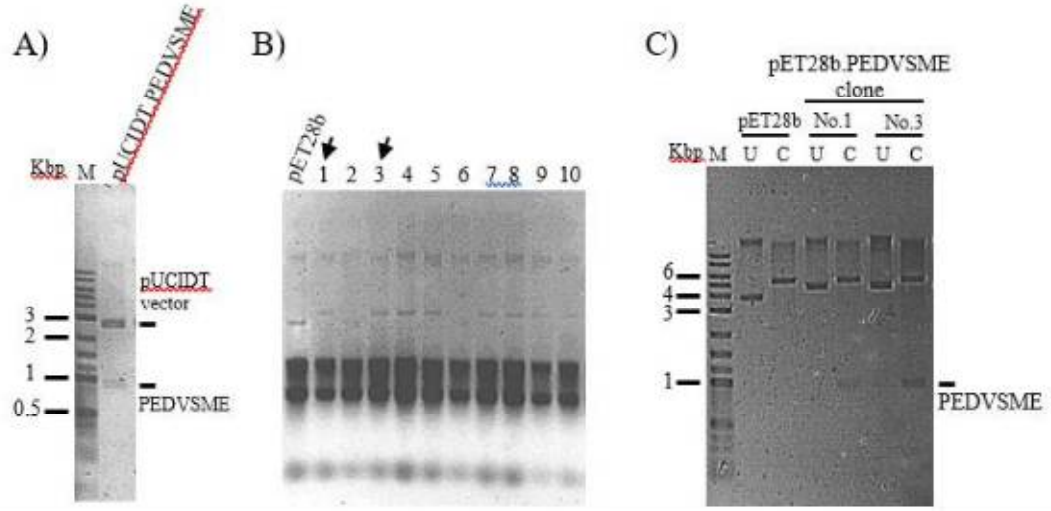


**Figure 1**. *PEDVSME* derivation, screening and confirmation of the pET28b.PEDVSME clones. (A) Derivation of the *PEDVSME* fragment from plasmid pUCIDT.PEDVSME using *Hind*III and *Not*I digestion. (B) Screening of recombinant clones using rapid size screening. Number represents clone number. (C) Confirmation of the *PEDVSME* gene presence in recombinant clones. The plasmids were double digested with *Hind*III and *Not*I. M represents 2-Log DNA ladder. U and C represent uncut and restriction enzyme-cut plasmid, respectively.

**Expression of the *PEDVSME* in *E. coli* strains**

As the PEDVSME immunogen is an engineered chimeric protein, conformation of the protein may not be optimal for solubilizing and being stable in the host cell. Thus, we studied the expression of this immunogen in two strains of *E. coli*: BL21(DE3) and BL21(DE3)pLysS. Recombinant *E. coli* clones were generated by transforming those 2 strains with plasmid pET28b.PEDVSME. Expression of the *PEDVSME* gene was then induced with IPTG. Following a 3-hour IPTG induction, protein expression was investigated using SDS-PAGE and Western blot. Interestingly, recombinant *E. coli* BL21(DE3) bearing *PEDVSME* gene grew considerably slow, compared to *E. coli* BL21(DE3)pLysS, particularly, after addition of IPTG.

When analyzed by SDS-PAGE, both soluble and inclusion body fractions obtained from strain BL21(DE3) was seen in a very low amount and majority of the protein was found at the size lower than 15 kDa (Figure 2A), indicating that most of the proteins are degraded. In contrast, the soluble and inclusion body fractions extracted from strain BL21(DE3)pLysS displayed normal pattern of proteins. *E. coli* strain BL21(DE3)pLysS bearing parental plasmid pET28b, which was used as a negative control, also showed normal protein pattern and amount. For soluble protein (supernatant fraction, S/N), a band of PEDVSME protein was observed in SDS-PAGE at the expected size of 38 kDa (Figure 2A). For inclusion body fraction, while the negative control sample showed only small amount of protein, the sample obtained from recombinant *E. coli* BL21(DE3)pLysS exhibited a protein at the expected size of 38 kDa together with other larger and smaller protein bands.

Western blot was performed to confirm the expression of the PEDVSME immunogen. The results showed that PEDVSME protein could not be detected in *E. coli* BL21(DE3) but highly expressed in *E. coli* BL21(DE3)pLysS (Figure 2B). Interestingly, the PEDVSME protein expressed by *E. coli* strain BL21(DE3)pLysS was majorly found in insoluble form (inclusion body) and only a small proportion was observed as soluble proteins. Cleaved products of the PEDVSME protein was also found in both soluble and inclusion body fractions, indicating instability of the protein in *E. coli* cell. Moreover, larger sizes of the PEDVSME protein were seen in both soluble and inclusion body fractions, suggesting interaction and aggregation of the protein within *E. coli* cell.

Altogether, under the control of T7 promoter and with IPTG induction, the gene encoding PEDVSME could be efficiently expressed by plasmid pET28b in prokaryotic *E. coli* host system. However, only *E. coli* strain BL21(DE3)pLysS was able to yield large amount of the protein, while strain BL21(DE3) could not tolerate the presence of the PEDVSME immunogen and underwent cell death.

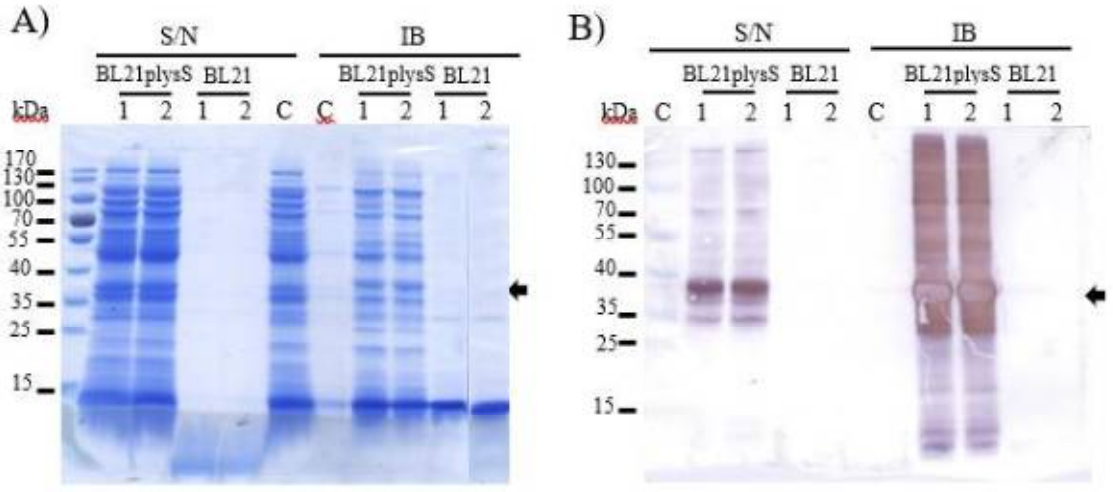


**Figure 2**. Expression of the *PEDVSME* immunogen in two *E. coli* strains. *E. coli* strain BL21(DE3)pLysS and BL21(DE3) bearing plasmid pET28b.PEDVSME and *E. coli* BL21(DE3)pLysS bearing parental pET28b were grown and induced with 1 mM IPTG for 3 hours. The cells were collected, lysed and separated into soluble fraction (supernatant, S/N) and inclusion body (IB). Expression of the PEDVSME protein was then investigated and confirmed using 10% SDS-PAGE (A) and Western blot (B). Predicted recombinant PEDVSME protein with the size of 38 kDa is depicted in an arrow. BL21pLysS and BL21 represent samples obtained from *E. coli* BL21(DE3)pLysS and BL21(DE3), respectively. 1 and 2 indicate clone number of each strain.

## DISCUSSION

Detected by SDS-PAGE and Western blot, PEDVSME was found to be highly expressed in *E. coli* strain BL21(DE3)pLysS, while it could not be detected in strain BL21(DE3). Moreover, *E. coli* BL21(DE3) expressing PEDVSME protein did not grow well and underwent cell death. This result suggests that a leaky expression may occur in strain BL21(DE3) before IPTG induction and PEDVSME may be toxic or induce cell stress, leading to growth retardation during culture and cell death after IPTG induction. This finding is

in agreement with the studies previously reported. Two studies have documented that over-production of the recombinant proteins in *E. coli* BL21(DE3) cannot be achieved due to the toxicity caused by leaky expression of the target products (George et al., 1994; Studier et al., 1990). In addition, expression of several membrane proteins and globular proteins, which was done in various strains of *E. coli*, showed that most of the *E. coli* strain BL21(DE3) host cells died after IPTG induction (Miroux & Walker, 1996).

Unlike strain BL21(DE3), strain BL21(DE3)pLysS contains the extra plasmid bearing the gene that encodes lysozyme and this lysozyme functions to inhibit the enzyme T7 polymerase, thus inhibiting the leaky expression before IPTG induction (Rosano & Ceccarelli, 2014; Studier & August, 1991). This feature of the strain BL21(DE3)pLysS may be beneficial to both survival of the host cell during culture and a high expression of the PEDVSME protein after IPTG induction. However, the PEDVSME protein was mainly found in inclusion body, which are insoluble aggregated. This may be due to an over-production of the protein within the cell; thus, only small amount of the protein can stay in soluble form because of limited space of the cytoplasm and the rest must be accumulated in insoluble aggregates (Gottesman, 1990; van den Berg et al., 1999). Furthermore, insoluble form known as inclusion body could be affected by incorrect disulfide bond formation, incorrect folding, low solubility of the protein, and the need of an essential translational modification (Rosano & Ceccarelli, 2014). These factors could also contribute to inclusion body formation of the PEDVSME protein.

## CONCLUSION

Production of the new immunogen, PEDVSME, was studied in two *E. coli* strains: BL21(DE3) and BL21(DE3)pLysS. The gene was under the control of IPTG-inducible T7 promoter in prokaryotic expression plasmid pET28b. Only strain BL21(DE3)pLysS could yield overproduction of the PEDVSME protein. However, the PEDVSME protein was mainly found in inclusion body. To further improve solubility, conformation and functional properties of the PEDVSME protein, cultivation parameters including time, temperature and inducer need to be studied.

## ACKNOWLEDGEMENT

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